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(54) Title: COMPOSITIONS AND METHODS TO TREAT NEURODEGENERATIVE DISORDERS

(57) Abstract: The present invention provides compositions comprising complexes of heat shock proteins non-covalently or covalently linked to antigens that display the antigenicity of antigens found in cells and tissues associated with the pathology of a neurodegenerative disease or disorder (such as Alzheimer's Disease). The compositions may be isolated from any tissue sources in which they exist, such as diseased human cells, non-human models for the disease or *in vitro* cultured cells that express neurodegenerative disorder-associated antigens. The invention further provides methods for the prevention and treatment of neurodegenerative diseases or disorders utilizing the compositions of the invention. The invention also provides kits comprising the compositions of the invention.

COMPOSITIONS AND METHODS TO TREAT NEURODEGENERATIVE DISORDERS

This invention was made with government support under grant numbers CA44786 and-CA64394 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. FIELD OF THE INVENTION

The present invention relates to compositions comprising complexes of heat shock proteins non-covalently or covalently bound to peptides associated with a neurodegenerative disorder such as Alzheimer's Disease. The invention further relates to methods of using the neurodegenerative disorder-associated heat shock protein-peptide complexes, including Alzheimer's Disease-associated heat shock protein-peptide complexes, in the prevention and treatment of neurodegenerative disorders such as Alzheimer's Disease.

2. BACKGROUND OF THE INVENTION

2.1. NEURODEGENERATIVE DISORDERS

Neuropsychiatric and neurodegenerative disorders (NDs) beginning to be understood at the molecular level. Strikingly similar pathologies commonly associated with the neurodegenerative disorders can be arrived at by a large number of different genetic mechanisms. For example, a pathogenic mutation in the prion gene results in both tangle and Lewy body pathologies of prion disease (Feany and Kickson, 1995, Am. J. Pathol. 146: 1388). Mutations in tau protein lead to dementia in frontotemporal dementia (Hutton et al., 1998, Nature 393: 702) in addition to neurofibrillary tangles; mutations in synuclein lead to the presence of Lewy bodies and Parkinson's disease (Polymeropoulos *et al.*, 1997, Science 276: 2045).

2.1.1. ALZHEIMER'S DISEASE

Alzheimer's Disease (AD) is a neurodegenerative disorder of the elderly that results in dementia and, ultimately, death. The physical alterations in the brains of diseased individuals are both intracellular, manifested as neurofibrillary tangles consisting of 10 nm

paired helical filaments (PHFs); and extracellular, manifested as amyloid plaques surrounding nerve terminals. Other physical changes may include microvascular amyloidosis and dystrophic cortical neurites (for a review on the pathological hallmarks of AD, see Sobow, 1996, Folia Neuropathol. 34:55-62).

Thus far, there is no concrete evidence as to whether the pathologic lesions are symptomatic or causative of AD. However, the components of the two main types of lesions are known. Neurofibrillary tangles consist of the intermediate filament protein Tau. In healthy neuronal tissue, Tau is an unphosphorylated protein but is found to be phosphorylated in PHFs. Amyloid plaques, also called senile plaques, consist of amyloid-β-peptide (Aβ), a product of the cleavage of an amyloid precursor protein (APP). In normal individuals, most of Aβ is in a 40-amino acid form; in addition, there exist minor amounts of Aβ that is 42 amino acids in length. In individuals with AD, the 42 amino acid form of Aβ predominates. The extent to which neurofibrillary tangles and amyloid plaques are present in the brain corresponds to the degree of senility caused by AD.

Many cases of AD result from a genetic predisposition to the disease, including most early onset AD. Early onset AD is almost exclusively familial AD (FAD) (reviewed by Price and Sisodia, 1998, Ann. Rev. Neurosci. 21: 479-505) and is associated with mutations in APP (on chromosome 21; Goate, et al., 1991, Nature, 349:704-706), presenilin 1 (PS1 on chromosome 14; Sherrington, et al., 1995, Nature 375:754-760) and presenilin 2 (PS2 on chromosome 1; Levy-Lahad, et al.,1995, Science 269:973-977), which together have been variably estimated to account for 10 to 40-50% of cases of the disease (Selkoe, 1999, Nature 399 Suppl.: A23-A31). The presenilins are membrane proteins having eight transmembrane domain and have been recently shown to be required for the processing of Notch protein (De Strooper *et al.*, 1999, Nature 398:518-522; Struhl and Greenwald, *ibid.*:522-525; Ye *et al.*, *ibid.*:525-529). Unlike mutations in PS1, mutations in PS2 are not completely penetrant and the age of onset of AD in carriers of PS1 mutations varies from 40 to 88 years (Renbaum and Levy-Lahad, 1998, Cell Mol. Life Sci. 54:910-919).

In addition to the genetic factors of FAD described above, it was noted in the 1960s and 1970s that individuals with Down Syndrome who live to the age of 40 generally develop the pathological changes associated with AD, although the clinical manifestations of the neuropathology are difficult to determine accurately due to the initially low levels of

mental functioning (reviewed by Karlinsky, J. Am. Geriatr. Soc. 34:728-734). The preponderance of AD in Down Syndrome was eventually linked to overexpression of APP after the genetic mapping of APP to human chromosome 21. It is thought that the higher levels of APP that result from a third copy of the gene encoding the protein lead to increased amyloid deposits which eventually lead to AD (reviewed by Hyman, 1992, Prog. Clin. Biol. Res. 379:123-142).

In late onset AD, no specific gene mutations are associated with the inheritance of AD; however, specific alleles of apolipoprotein $\epsilon 4$ and $\alpha 2$ macroglobulin are associated with increased risk for AD.

Thus, a common theme in AD pathology is the production of aberrant structures that are not normally found in healthy brains. This observation is supported by the finding that PHFs are associated with ubiquitin, which would normally lead to the degradation of the associated molecule but in AD does not do so.

There is no single test that can accurately diagnose AD. However, using a combination of medical history, mental status evaluation, physical examination, neurological examination, laboratory tests (*e.g.* blood and urine tests; MRI or CT scans) and other psychiatric evaluations, it is possible to determine with an 85-90% degree of accuracy whether a patient has AD (see the web site of the Alzheimer's Association at www.alz.org). Molecular methods of diagnosing AD have been disclosed but not yet put to use on a commercial level. For example, diagnostic tests based on the presence of Tau peptides in a patient or subject's blood (U.S. Patent No. 5,492,812) or the presence of p33 (Annexin V) proteins in a patient or subject's cerebrospinal fluid (U.S. Patent No. 5,849,600) have been proposed. Additionally, genetic testing, for example to determine which allele(s) of APP, PS1 or PS2 an individual carries, can be carried out to determine the individual's risk of developing AD.

At the present time, there are no means by which AD is prevented or cured. Immunization of mice with $A\beta$ antigens was shown to prevent the formation of amyloid plaques in the mice (Schenk et al., 1999, Nature 400:173-177). In humans, it has been noted that hormone replacement therapy that provides estrogen to post-menopausal women provides a beneficial effect to the development of AD, although the benefits are determined by the genetic background of the individual (van Duijn, 1999, Maturitas 31:201-205). Additionally, since the cognitive impairment associated with AD is at least in part attributed

to cholinergic deficits, several acetylcholinesterase inhibitors (AchEIs), for example tacrine hydrochloride (Cognex™; Warner Lambert), have been used in an attempt to prevent the degradation of acetylcholine and retard AD-associated neurodegeneration, the use having met with only moderate success (Krall et al., 1999, Ann. Pharmacother. 33:441-450). Other putative treatments for AD are currently being tested in clinical trials, for example anti-inflammatory drugs and α-tocopherol (Shadlen and Larson, 1999, Postgrad. Med. 105:109-118). However, there remains a great need for novel modalities for prevention of the fourth leading cause of death in developed countries in those at high risk of acquiring the disease, as determined by genetic testing, and for treatment of AD in patients who have developed the disease.

2.2. HEAT SHOCK PROTEINS

Heat shock proteins (hsps), also referred to interchangeably as stress proteins, were first identified as proteins synthesized by a cell in response to heat shock. To date, five major classes of hsps have been identified, based on the molecular weight of the family members. These classes are called shsps (small heat shock proteins), Hsp60, Hsp70, Hsp90, and Hsp100, where the numbers reflect the approximate molecular weight of the hsps in kilodaltons.

Many hsps have been found to be induced in response to stressful stimuli other than heat, including nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8: 401-420; Craig, 1993, Science 260: 1902-1903; Gething *et al.*, 1992, Nature 355: 33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22: 631-677). Heat shock proteins are highly conserved proteins. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from excoriates (Bardwell *et al.*, 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 848-852). The Hsp60 and Hsp90 families also show similarly high levels of intra-family conservation (Hickey *et al.*, 1989, Mol. Cell. Biol. 9: 2615-2626; Jindal, 1989, Mol. Cell. Biol. 9: 2279-2283).

Hsps are involved not only in cellular protection against adverse conditions, but are also involved in essential biochemical and immunological processes in unstressed cells. For example, hsps are involved in various kinds of chaperoning functions. Members of the Hsp70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic

reticulum are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells (Lindquist *et al.*, 1988, Ann. Rev. Genetics 22: 631-677). A number of proteins thought to be involved in chaperoning are residents of the endoplasmic reticulum (ER) lumen, for example, protein disulfide isomerase (PDI; Gething *et al.*, 1992, Nature 355: 33-45), Grp94 or ERp99 (Sorger & Pelham, 1987, J. Mol. Biol. 194: 991-94) which is related to Hsp90, and Grp78 or BiP, which is related to Hsp70 (Munro *et al.*, 1986, Cell 46: 291-300; Haas & Webl, 1983, Nature 306: 387-389). These proteins are known to bind a variety of mutant, unfolded, incompletely glycosylated proteins (Machamer *et al.*, 1990, J. Biol. Chem. 65: 6879-6883; Gething *et al.*, 1986, Cell 46: 939-950).

The roles of hsps as protein chaperones extends to forming complexes with antigenic molecules and presenting them to the immune system, for example in cancer cells (Srivastava et al., 1996, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich et al., 1996, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with hsp complexes derived from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). Methods and compositions for the prevention and treatment of cancer and/or infectious disease by stimulating the immune response using heat shock proteins are disclosed in U.S. Patent Nos. 5,750,119; 5,837,251; 5,830,464; and 5,935,576. Optionally, the hsp-antigen complexes may be used in combination with adoptive immunotherapy, *i.e.* in combination with antigen presenting cells that contain the hsp-antigen complexes (*see e.g.* U.S. Patent Nos. 5,830,464 and 5,985,270).

Citation or identification of any reference herein shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a ND or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a ND. The ND includes but is not limited to Alzheimer's Disease, age-related loss of cognitive

function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

In certain embodiments, the invention provides compositions comprising a purified population of complexes bound to endogenous antigenic molecules, which population is purified from mammalian tissues or cells, which tissues or cells display a pathology associated with AD or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with AD. In certain embodiments of the invention, the purified population of complexes bound to endogenous antigenic molecules is from an individual with AD, for example from brain tissue or other AD-associated tissue such as pancreatic tissue. In another embodiment of the invention, the purified population of complexes bound to endogenous antigenic molecules is isolated from brain or pancreatic tissue of an individual with Trisomy 21. In certain modes of the embodiment, the tissue is isolated post-mortem from a cadaver of an individual with AD or Trisomy 21. In yet others embodiment, the purified population of complexes bound to endogenous antigenic molecules is isolated from a culture of olfactory neurons from an individual with AD or Trisomy 21.

In other embodiments of the invention, the purified population of complexes bound to endogenous antigenic molecules is from an AD model organism. In one embodiment, the organism is a rat which has received infusions of Aβ and TGF-β. In another embodiment of the invention, the AD model organism is a transgenic mouse. In a preferred mode of the embodiment, the mouse expresses human APP V717F and optionally another AD-associated protein. In yet another embodiment, the AD model organism is a non-human primate.

In yet other embodiments of the invention, the purified population of complexes bound to endogenous antigenic molecules is from a tissue or cell culture model system for AD. In a certain specific embodiment of the invention, the tissue or cell culture

model system is selected from the group consisting of immortalized murine Trisomy 16 neuronal cells, mouse neuroblastoma cell line S20Y, and mouse embryonal carcinoma cell line P19.

In yet other embodiments of the invention, the purified population of complexes bound to endogenous antigenic molecules is from cells associated with amyloid plaques or cells containing neurofibrillary tangles.

The compositions of the invention comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a ND or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a ND comprise hsp70, hsp90, gp96, calreticulin (for the ability of calreticulin-anitgenic molecule complexes to elicit an immune response, see Basu and Srivastava, 1999, J. Exp. Med. 189:797-202) or a combination thereof.

The invention further provides pharmaceutical composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

The present invention provides methods of treating or preventing a neurodegenerative disorder in a mammal comprising administering to the mammal a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a ND or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a ND. The neurodegenerative disorder includes but is not limited to Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure

disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder. In certain embodiments, the composition comprises hsp70, hsp90, gp96, calreticulin, or a combination thereof.

In certain embodiments, the present invention provides methods of preventing or treating AD in a human subject comprising administering to the subject a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with AD or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with AD. In a preferred embodiment, the AD-associated heat shock protein-peptide complex is autologous. In certain embodiments, the composition is administered intrathecally or intradermally.

The present invention further provides a kit comprising in one or more separate containers a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a ND or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a ND.

The invention further provides a method of treating or preventing a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom treatment or prevention of a neurodegenerative disorder is desired comprising isolating or culturing a mammalian tissue or cell that displays a pathology associated with a neurodegenerative disorder or expresses an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder; recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules; and administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder. Optionally, the complexes are treated with a cross-linking reagent prior to administeration.

The invention further provides a method for eliciting in an individual an immune response against a tissue or cell which displays a pathology associated with a

neurodegenerative disorder comprising administering to the individual a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder.

The invention further provides a method for eliciting in an individual an immune response against an antigen associated with a neurodegenerative disorder comprising administering to the individual a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said population being purified from mammalian tissues or cells and comprising a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

3.1. ABBREVIATIONS AND DEFINITIONS

As used herein, the following terms and phrases shall have the meanings indicated below:

AD: Alzheimer's Disease

APP: Amyloid Precursor Protein

Aβ40: The 40 kilodalton cleavage product of APP; the prevalent cleavage product of APP in healthy individuals.

Aβ42: The 42 kilodalton cleavage product of APP; the prevalent cleavage product of APP in individuals with AD.

CSF: Cerebrospinal fluid

FAD: Familial AD

hsp: heat shock protein

ND: Neurodegenerative disorder or disease

ND-associated antigen: a proteinaceous molecule whose expression or overexpression (by increased transcription of the encoding gene, increased translation of the encoding RNA, increased or aberrant processing of a precursor protein, or increased or aberrant posttranslational modification of the molecule or a precursor thereof) is associated with a neurodegenerative disorder or disease. An ND-associated antigen is expressed in mammalian tissues or cells which display a pathology associated with a neurodegenerative

disorder and/or displays the antigenicity of an antigen associated with a neurodegenerative disorder.

NFT: Neurofibrillary tangle

PS1: Presenilin 1

PS2: Presenilin 2

PHF: Paired helical filament, a type of neuropathological structure associated with AD.

4. DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions for the prevention and treatment of neurodegenerative diseases or disorders (NDs), such as Alzheimer's Disease (AD), are provided by this invention. The methods comprise the administration of hsp-peptide complexes isolated from ND-associated tissues or cells. As used herein, ND-associated tissues or cells refers to tissues or cells which display the antigenicity of an antigen associated with a neurodegerative disorder. For example, AD-associated tissues or cells include but are not limited to the brain lesions or pathologic structures (such as amyloid plaques and neurofibrillary tangles that are associated with Alzheimer's Disease), brain tissue of rodent or non-human primate models of Alzheimer's Disease, cell culture cells that overexpress AD antigens, or non-brain tissue from individuals with AD or from individuals with a genetic predisposition to the disease. Such AD-associated tissues or cells are described in Section 4.1, *infra*.

The ND-associated hsp-peptide complexes of the invention include any complex derived from a neurodegenerative disease or disorder tissue or a model cell or tissue thereof containing an hsp and a peptide that is capable of stimulating an immuno-protective or -therapeutic response against the neurodegenerative disease. The peptides of the complexes can be noncovalently or covalently associated with the hsp. The complexes are purified in a non-covalent form and, optionally, their components covalently coupled after they are purified (see Section 4.5, *infra*). In a specific embodiment, the ND-associated hsp-peptide complexes used in accordance with the invention comprise a human hsp.

The hsps that can be used according to the present invention include but are not limited to, gp96, hsp90, hsp70, and calreticulin, either alone or in combination with each other.

Preferred complexes include, but are not limited to, ND-associated hsp90-peptide complexes, ND-associated hsp70-peptide complexes, ND-associated hsp60-peptide complexes and combinations thereof. For example, an hsp called gp96 which is present in the endoplasmic reticulum of eukaryotic cells and is related to the cytoplasmic hsp90s (*i.e.*, is a member of the hsp90 family) can be used to generate an effective therapeutic composition containing a gp96-peptide complex.

Heat shock proteins, which are also referred to interchangeably herein as stress proteins, useful in the practice of the instant invention can be selected from among any cellular protein that satisfies any one of the following criteria. A heat shock protein is characterized by having its intracellular concentration increase when a cell is exposed to a stressful stimuli, by being capable of binding other proteins or peptides, by being capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH, or by having at least 35% homology with any cellular protein having any of the above properties.

The first stress proteins to be identified were the heat shock proteins (hsps). As their name implies, hsps are synthesized by a cell in response to heat shock. To date, five major classes of hsps have been identified, based on the molecular weight of the family members. These classes are called shsps (small heat shock proteins), Hsp60, Hsp70, Hsp90, and Hsp100, where the numbers reflect the approximate molecular weight of the hsps in kilodaltons. Mammalian hsp90 and gp96 each are members of the hsp90 family. Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens. (See Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8:401-420; Craig, 1993, Science 260:1902-1903; Gething, et al., 1992, Nature 355:33-45; and Lindquist, et al., 1988, Annu. Rev. Genetics 22:631-677). It is contemplated that complexes of hsps/stress proteins from any of these three families can be used in the practice of the instant invention.

The major hsps can accumulate to very high levels in stressed cells, but they occur at low to moderate levels in cells that have not been stressed. For example, the highly inducible mammalian hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch, et al., 1985, J.

Cell. Biol. 101:1198-1211). In contrast, hsp90 and hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai, et al., 1984, Mol. Cell. Biol. 4:2802-10; van Bergen en Henegouwen, et al., 1987, Genes Dev. 1:525-31).

Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the hsp70 from *E. coli* has about 50% amino acid sequence identity with hsp70 proteins from excoriates (Bardwell, et al., 1984, Proc. Natl. Acad. Sci. 81:848-852). The hsp60 and hsp90 families also show similarly high levels of intra families conservation (Hickey, et al., 1989, Mol. Cell. Biol. 9:2615-2626; Jindal, 1989, Mol. Cell. Biol. 9:2279-2283). In addition, it has been discovered that the hsp60, hsp70 and hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus. The purification of stress proteins belonging to these three families is described below.

The sections 4.1 and 4.6 which follow illustrate embodiments of the present invention that pertain to Alzheimer's Disease. A person of skill in the art can recognize that the same principles used to identify sources of AD-associated hsp-peptide complexes and methods of monitoring the progress of AD can be applied to other neurodegenerative disorders, including but not limited to those cited in Section 4.8, *infra*.

4.1. SOURCES OF AD-ASSOCIATED HSP-PEPTIDE COMPLEXES

Sources from which the AD-associated hsp-peptide complexes of the present invention include, but are not limited to, human tissues, tissues from non-human AD model organisms, and *in vitro* cell cultures. For the embodiments of the invention in which the AD-associated hsp-peptide complexes are produced from human tissues, the preferred type of tissue is brain tissue, including cells and pathological structures, for example from

cadavers of AD patients. Other tissue type that express AD-associated antigens, such as the pancreas, can also be used. In another embodiment, brain tissue as well as other tissue types from rodents or other mammals with AD-like pathologies can be used. In yet another embodiment, cultured cells that may contain hsp-peptide complexes comprising AD-associated antigens complexed to endogenous or recombinantly expressed hsps, similar to those hsp-peptide complexes found in AD brains, are used. In a specific embodiment, the cultured cells overexpress hsps by recombinant methods (*e.g.* as described in WO 97/10000) to improve the recovery of AD-associated antigens.

In various embodiments, the AD-associated hsp-peptide complexes of the invention can be autologous to the patient, *i.e.* the complexes are derived originally from the patient to whom they are administered. In an alternative embodiment, the complexes are non-autologous.

4.1.1. HUMAN TISSUE

In certain preferred aspects of the invention, the AD-associated hsp-complexes of the invention are obtained from human brain tissue. The brain tissue can originate from a symptomatic AD patient, *e.g.* a senile adult diagnosed with AD; an asymptomatic AD patient, for example an individual who is genetically predisposed to AD who has developed the pathological structures but not the clinical manifestations of AD; or an individual with trisomy 21. The brain tissue may be obtained ante mortem (for example from a biopsy) or post mortem. The AD-associated hsp-complexes may be prepared from whole brain tissue or, in a certain embodiment, from specific anatomical structures containing cells with neurofibrillary tangles or cells that secrete amyloid plaques.

In addition to the isolation of AD-associated hsp-complexes from brain tissue, the complexes can be isolated from other organs such as but not limited to the pancreas, the kidney, or skeletal muscle. These organs in particular have been noted to contain pathological structures similar to those observed in AD brains. For example, overexpression of a C-terminal APP fragment in transgenic mice results in Aβ deposits and amyloid fibrils in the brains, kidneys and pancreases of the mice (Kawarabayashi et al., 1996, Neurolbiol. Aging 17:215-222; Shoji et al., 1996, Gerontology 42 Suppl.:48-56), with especially high deposition of Aβ in the acinar cells and interstitial macrophages of pancreas. Expression of the same fragment also results in Aβ deposits in the skeletal

muscles of the mice (Fukuchi et al., 1998, Am. J. Pathol. 153:1687-1693). Thus, these tissues can serve as good sources of AD-associated hsp-peptides complexes in subjects with APP abnormalities, such as AD patients, asymptomatic individuals having APP-related FAD, or individuals with trisomy 21. In a preferred embodiment, the tissue is obtained post mortem, although the tissue can also be obtained from tissue biopsies ante mortem or from tissue explants.

4.1.2. TISSUE FROM NON-HUMAN AD MODEL ORGANISMS

In another embodiment of the invention, transgenic mice created as models for AD are used as sources of tissues (*e.g.* brain, pancreas, kidneys, muscle) for the isolation of AD-associated hsp-peptide complexes. Several transgenic mouse models of AD have been described in the scientific literature, most of which express various forms of AD-associated antigens. The first successful transgenic mouse models of AD harbored the APP mutant V717F, a form of APP that causes FAD. The APP V717F-expressing mice displayed AD-like neuropathologies such as Aβ deposits and neuritic plaques (Games et al., 1995, Nature 373:523-527) although they fail to acquire neurofibrillary tangles (Chen et al., 1998, Prog. Brain Res. 117:327-334). In contrast, transgenic mice that express the longest human brain tau protein isoform accumulate paired helical filaments in their brain tissues but not neurofibrillary tangles (Gotz et al., 1995, EMBO J. 14:1304-1313). Other transgenic mouse models for AD include but are not limited to mice expressing or overexpressing the APP isoforms APP695, APP751, APP770, and mice which overexpress APP or presenilin proteins having mutations corresponding to those seen in patients with FAD (for example PS1 I213T; Nakano et al., 1999, Eur. J. Neurosci:2577-2581). In a preferred specific embodiment, the transgenic mice from which the AD-associated hsp-peptide complexes of the invention are obtained are mice that have been cross-bred to overexpress more than one of the AD-associated proteins described above.

In an alternative embodiment, the mice from which AD-associated hsp-peptide complexes are isolated are trisomy 16 mutant mice. Mouse chromosome 16 contains the mouse counterpart of APP and trisomy 16 mice serve as a model system for Down's syndrome (Epstein et al., 1985, Ann. NY Acad. Sci. 450:157-68). However, the

mice die during the third week of gestation. Accordingly, the AD-associated hsp-peptide complexes of the invention are isolated from fetal tissue.

It has also been shown that rats that have received intraventricular infusions of Aβ together with neuropil injection of TGFβ develop Aβ-positive plaque-like deposits (Frautschy et al., 1996, Neurobiol. Aging 17:311-321). The deposits or the tissue surrounding the deposits can be isolated for hsp-peptide preparations as described in Section 4.3, *infra*, and used according to the methods of the present invention.

In another embodiment of the invention, the AD-associated hsp-peptide complexes of the invention are isolated from non-human primates. The non-human primate brain has been shown to develop the same classes of pathological structures seen in the human brain as a result of aging or AD (Voytko, 1998, Lab. Animal Sci. 48:611-617), making them excellent sources of AD-associated hsp-peptide complexes. In a certain embodiment, the AD-associated hsp-peptide complexes of the invention are isolated from non-human primates whose brains have been infused with Aβ and TGFβ, as described *supra.*

In another embodiment of the invention, the AD-associated hsp-peptide complexes of the invention are isolated from *in vitro* cultured cells that express antigens associated with AD. The complexes can be isolated from primary culture cells or from cells of immortalized cell lines. Any cell line that has been characterized to express at least one AD-associated antigen can be used to isolate the hsp-peptide complexes of the present invention. The cells lines can be but are not limited to being of central nervous system, kidney, pancreas or muscle origin. For more efficient recovery of the complexes of the invention, the cells can be manipulated to overexpress an hsp molecule. For example, the cells may be transfected with an expression vector comprising an hsp70, hsp90 or gp96 open reading frame and grown under conditions that allow for the expression of corresponding protein prior to hsp-peptide complex isolation.

Human olfactory neurons isolated from the central nervous system can be made to divide in cell culture (U.S. Patent No. 5,869,266). Thus, they provide replicable cells that, when isolated from an AD individual, are likely to express the full repertoire of AD-associated antigens, making them an ideal renewable source for the isolation of AD-associated hsp-peptide complexes, particularly if they are made to overexpress hsps, for

example by transfecting into the neurons an expression vector harboring an open reading frame encoding an hsp molecule.

In one embodiment, the neurons are isolated from a cadaver of an AD or trisomy 21 individual. In another embodiment, the neurons originate from the patient or subject to whom the compositions of the invention are administered, for example by way of a tissue biopsy, such that the complexes are autologous to said patient or subject.

Several neuronal cell lines have been immortalized from trisomy 16 mice (Frederiksen et al., 1996, Ann. NY Acad. Sci. 777:415-420). These can be used as sources of AD-associated hsp-complexes.

In a specific embodiment, S20Y, a murine cholinergic neuroblastoma cell line that has been shown to become immunoreactive to PHF antibodies when grown under certain conditions (Ko et al., 1990, Acta Neuropathol. (Berl.) 81:30-40), can be used to isolate the complexes. In another specific embodiment, the cell line used to isolate AD-associated hsp-peptide complexes is P19. P19 is a murine embryonal carcinoma cell line that differentiates into cholinergic neurons upon retinoic acid treatment, resulting in high levels of APP and Tau protein expression (Fukuchi et al., 1992, J. Neurochem., 58:1863-1873).

4.2. ISOLATION OF ND-ASSOCIATED TISSUE

ND-associated tissue from humans or animals can be isolated by standard methods in the art, *e.g.* dissection. For example, simply using a scalpel, it is possible to dissect out specific anatomical structures of the brain, for example to separate grey matter from white matter or the cortex from subcortical nuclei. Any other technique known in the art for isolation of the tissue may be used.

4.3. ND-ASSOCIATED HSP-PEPTIDE COMPLEXES

The methods described in the sections that follow can be used to isolate the ND-associated hsp-peptide complexes of the invention from cells that express hsps endogenously as well as from cells that express higher levels of hsps through recombinant means. Recombinant expression of hsps is described in Section 4.4, *infra.*

Preferably, the ND-associated hsp-peptide complexes of the invention are used in purified form, preferably to apparent homogeneity as viewed on an SDS-PAGE gel, or to at least 60%, 70%, 80%, or 90% of total protein.

4.3.1. PREPARATION AND PURIFICATION OF GP96-PEPTIDE COMPLEXES

A procedure that can be used, presented by way of example and not limitation, is as follows:

A pellet of eukaryotic cells (*e.g.*, from the Alzheimer diseased brain of a cadaver, or more specifically the regions of the brain containing amyloid plaques and/or neurofibrillary tangles) is resuspended in 3 volumes of buffer consisting of 30mM sodium bicarbonate buffer (pH 7.5) and 1mM PMSF and the cells allowed to swell on ice 20 minutes. The cell pellet then is homogenized in a Dounce homogenizer (the appropriate clearance of the homogenizer will vary according to each cell type) on ice until >95% cells are lysed.

The lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other debris. The supernatant from this centrifugation step then is recentrifuged at 100,000Xg for 90 minutes. The gp96-peptide complex can be purified either from the 100,000Xg pellet or from the supernatant.

When purified from the supernatant, the supernatant is diluted with equal volume of 2X lysis buffer and the supernatant mixed for 2-3 hours at 4°C with Con A-Sepharose® (Pharmacia, Inc., Sweden) equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. Then, the slurry is packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then, the column is washed with 1/3 column bed volume of 10% α-methyl mannoside (α-MM) dissolved in PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}, the column sealed with a piece of parafilm, and incubated at 37°C for 15 minutes. Then the column is cooled to room temperature and the parafilm removed from the bottom of the column. Five column volumes of the α-MM buffer are applied to the column and the eluate analyzed by SDS-PAGE. Typically the resulting material is about 60-95% pure, however this depends upon the cell type and the tissue-to-lysis buffer ratio used. Then the sample is applied to a Mono Q® FPLC ion-exchange chromatographic column (Pharmacia, Inc., Piscataway, NJ) equilibrated with a buffer containing 5mM sodium phosphate, pH 7. The

proteins are then eluted from the column with a 0-1M NaCl gradient. The gp96 fraction elutes between 400mM and 550mM NaCl.

The procedure, however, can be modified by two additional steps, used either alone or in combination, to consistently produce apparently homogeneous gp96-peptide complexes. One optional step involves an ammonium sulfate precipitation prior to the Con A purification step and the other optional step involves DEAE-Sepharose® purification after the Con A purification step but before the Mono Q® FPLC step.

In the first optional step, the supernatant resulting from the 100,000Xg centrifugation step is brought to a final concentration of 50% ammonium sulfate by the addition of ammonium sulfate. The ammonium sulfate is added slowly while gently stirring the solution in a beaker placed in a tray of ice water. The solution is stirred from about 1/2 to 12 hours at 4°C and the resulting solution centrifuged at 6,000 rpm (Sorvall SS34 rotor). The supernatant resulting from this step is removed, brought to 70% ammonium sulfate saturation by the addition of ammonium sulfate solution, and centrifuged at 6,000 rpm (Sorvall SS34 rotor). The resulting pellet from this step is harvested and suspended in PBS containing 70% ammonium sulfate in order to rinse the pellet. This mixture is centrifuged at 6,000 rpm (Sorvall SS34 rotor) and the pellet dissolved in PBS containing 2mM Ca^{2+} and Mg^{2+}. Undissolved material is removed by a brief centrifugation at 15,000 rpm (Sorvall SS34 rotor). Then, the solution is mixed with Con A Sepharose® and the procedure followed as before.

In the second optional step, the gp96 containing fractions eluted from the Con A column are pooled and the buffer exchanged for 5mM sodium phosphate buffer, pH 7, 300mM NaCl by dialysis, or preferably by buffer exchange on a Sephadex® G25 column (Pharmacia, Inc., Sweden). After buffer exchange, the solution is mixed with DEAE-Sepharose® previously equilibrated with 5mM sodium phosphate buffer, pH 7, 300mM NaCl. The protein solution and the beads are mixed gently for 1 hour and poured into a column. Then, the column is washed with 5mM sodium phosphate buffer, pH 7, 300mM NaCl, until the absorbance at 280nM drops to baseline. Then, the bound protein is eluted from the column with five volumes of 5mM sodium phosphate buffer, pH 7, 700mM NaCl. Protein containing fractions are pooled and diluted with 5mM sodium phosphate buffer, pH 7 in order to lower the salt concentration to 175mM. The resulting material then is applied to the Mono Q® FPLC column (Pharmacia) equilibrated with 5mM sodium phosphate

buffer, pH 7 and the protein that binds to the Mono Q® FPLC column (Pharmacia) is eluted as described before.

It is appreciated, however, that one skilled in the art can assess, by routine experimentation, the benefit of incorporating the second optional step into the purification protocol. In addition, it is appreciated also that the benefit of adding each of the optional steps will depend upon the source of the starting material.

When the gp96 fraction is isolated from the 100,000Xg pellet, the pellet is suspended in 5 volumes of PBS containing either 1% sodium deoxycholate or 1% octyl glucopyranoside (but without the Mg^{2+} and Ca^{2+}) and incubated on ice for 1 hour. The suspension is centrifuged at 20,000Xg for 30 minutes and the resulting supernatant dialyzed against several changes of PBS (also without the Mg^{2+} and Ca^{2+}) to remove the detergent. The dialysate is centrifuged at 100,000Xg for 90 minutes, the supernatant harvested, and calcium and magnesium are added to the supernatant to give final concentrations of 2mM, respectively. Then the sample is purified by either the unmodified or the modified method for isolating gp96-peptide complex from the 100,000Xg supernatant, see above.

The AD-associated gp96-peptide complexes can be purified to apparent homogeneity using this procedure. About 10-20μg of AD-associated gp96-peptide complex can be isolated from 1g cells or tissue.

4.3.2. PREPARATION AND PURIFICATION OF HSP 70-PEPTIDE COMPLEXES

The purification of hsp70-peptide complexes has been described previously, see, for example, Udono et al., 1993, J. Exp. Med. 178:1391-1396. A procedure that can be used, presented by way of example but not limitation, is as follows:

Initially, cells (*e.g.*, from the Alzheimer-diseased brain of a cadaver, or more specifically the regions of the brain containing amyloid plaques and/or neurofibrillary tangles) are suspended in 3 volumes of 1X lysis buffer consisting of 5mM sodium phosphate buffer, pH 7, 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells can be lysed by mechanical shearing and in this approach the cells typically are resuspended in

30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000Xg for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose® equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X lysis buffer prior to mixing with Con A Sepharose®. The supernatant is then allowed to bind to the Con A Sepharose® for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q® FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20mM to 500mM NaCl gradient and eluted fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-hsp70 antibody (such as from clone N27F3-4, from StressGen, Victoria, British Columbia, Canada).

Fractions strongly immunoreactive with the anti-hsp70 antibody are pooled and the hsp70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex® G25 column (Pharmacia). If necessary the hsp70 preparation thus obtained can be repurified through the Mono Q® FPLC column as described above.

The hsp70-peptide complex can be purified to apparent homogeneity using this method. Typically 1mg of hsp70-peptide complex can be purified from 1g of cells/tissue.

The present invention further describes a rapid method for purification of hsp70-peptide complexes. This improved method comprises contacting cellular proteins with ADP or a nonhydrolyzable analog of ATP affixed to a solid substrate, such that hsp70 in the lysate can bind to the ADP or nonhydrolyzable ATP analog, and eluting the bound

hsp70. A preferred method uses column chromatography with ADP affixed to a solid substratum (*e.g.*, ADP-agarose). The resulting hsp70 preparations are higher in purity and devoid of contaminating peptides. The hsp70 yields are also increased significantly by about more than 10 fold. Alternatively, chromatography with nonhydrolyzable analogs of ATP, instead of ADP, can be used for purification of hsp70-peptide complexes.

By way of example but not limitation, purification of hsp70-peptide complexes by ADP-agarose chromatography is carried out as follows:

500 million cells (*e.g.*, from liver, spleen, or any other suitable organ) are homogenized in hypotonic buffer and the lysate is centrifuged at 100,000Xg for 90 minutes at 4°C. The supernatant is applied to an ADP-agarose column. The column is washed in buffer and is eluted with 5 column volumes of 3 mM ADP. The hsp70-peptide complexes elute in fractions 2 through 10 of the total 15 fractions which elute. The eluted fractions are analyzed by SDS-PAGE. The AD-associated hsp70-peptide complexes can be purified to apparent homogeneity using this procedure.

4.3.3. PREPARATION AND PURIFICATION OF HSP 90-PEPTIDE COMPLEXES

A procedure that can be used to prepare hsp90-peptide complexes, presented by way of example and not limitation, is as follows:

Initially, cells (*e.g.*, from the Alzheimer diseased brain of a cadaver, or more specifically the regions of the brain containing amyloid plaques and/or neurofibrillary tangles) are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells can be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 minutes and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1,000Xg for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000Xg for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose® equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells

are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose®. The supernatant is then allowed to bind to the Con A Sepharose® for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 250 mM NaCl, 1 mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 minutes. Then the resulting supernatant is harvested and applied to a Mono Q® FPLC column equilibrated with a buffer containing 20 mM sodium phosphate, pH 7.4, 1 mM EDTA, 250 mM NaCl, 1 mM PMSF. The proteins are then eluted with a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the hsp90-peptide complexes identified by immunoblotting using an anti-hsp90 antibody such as 3G3 (Affinity Bioreagents). AD-associated hsp90-peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200 μg of AD-associated hsp90-peptide complex can be purified from 1g of cells or tissue.

4.4. RECOMBINANT EXPRESSION OF HSPS

4.4.1. HSP SEQUENCES

In certain embodiments of the present invention, ND-associated hsp-peptide complexes are prepared from cells that express higher levels of hsps through recombinant means. Amino acid sequences and nucleotide sequences of many hsps are generally available in sequence databases, such as GenBank. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. Such nucleotide sequences of non-limiting examples of hsps that can be used for the compositions, methods, and for preparation of the hsp peptide-complexs of the invention are as follows: human hsp70, Genbank Accession No. M24743, Hunt *et al.*, 1995, Proc. Natl. Acad. Sci. U.S.A., 82: 6455-6489; human Hsp90, Genbank Accession No. X15183, Yamazaki *et al.*, Nucl. Acids Res. 17: 7108; human gp96: Genbank Accession No. X15187, Maki *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 5658-5562; human BiP: Genbank Accession No. M19645; Ting *et al.*, 1988, DNA 7: 275-286; human Hsp27,

Genbank Accession No. M24743; Hickey *et al.*, 1986, Nucleic Acids Res. 14: 4127-45; mouse Hsp70: Genbank Accession No. M35021, Hunt *et al.*, 1990, Gene 87: 199-204; mouse gp96: Genbank Accession No. M16370, Srivastava *et al.*, 1987, Proc. Natl. Acad. Sci. U.S.A. 85: 3807-3811; and mouse BiP: Genbank Accession No. U16277, Haas *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85: 2250-2254. Due to the degeneracy of the genetic code, the term "hsp gene", as used herein, refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode the hsp.

Once the nucleotide sequence encoding the hsp of choice has been identified, the nucleotide sequence, or a fragment thereof, can be obtained and cloned into an expression vector for recombinant expression. The expression vector can then be introduced into a host cell for propogation of the hsp. Methods for recombinant production of hsps are described in detail herein.

The DNA may be obtained by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library") using standard molecular biology techniques (see *e.g.*, Methods in Enzymology, 1987, volume 154, Academic Press; Sambrook *et al.* 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, New York; and Current Protocols in Molecular Biology, Ausubel et al. (eds.), Greene Publishing Associates and Wiley Interscience, New York, each of which is incorporated herein by reference in its entirety). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the hsp gene should be cloned into a suitable vector for propagation of the gene.

In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of a related or homologous hsp. PCR is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, *e.g.*, by use of a thermal cycler and Taq polymerase (Gene Amp®). The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding an hsp of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding open reading fram. Alternatively, an hsp gene sequence can be cleaved at appropriate sites with restriction

endonuclease(s) if such sites are available, releasing a fragment of DNA encoding the hsp gene. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1: 277-278). The DNA fragment that encodes the hsp is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained.

In an alternative embodiment, for the molecular cloning of an hsp gene from genomic DNA, DNA fragments are generated to form a genomic library. Since some of the sequences encoding related hsps are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to a labeled probe (Benton and Davis, 1977, Science 196: 180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72: 3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify an appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map.

Alternatives to isolating the hsp genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or synthesizing a cDNA to the mRNA which encodes the hsp. For example, RNA for cDNA cloning of the hsp gene can be isolated from cells which express the hsp. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to the hsp is available, the hsp may be identified by binding of a labeled antibody to the hsp-synthesizing clones.

Other specific embodiments for the cloning of a nucleotide sequence encoding an hsp, are presented as examples but not by way of limitation, as follows: In a specific embodiment, nucleotide sequences encoding an hsp can be identified and obtained by hybridization with a probe comprising a nucleotide sequence encoding hsp under conditions of low to medium stringency. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications:

0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson *et al.*, 1978, J. Biol. Chem. 253: 6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19: 423-463; Hill *et al.*, 1987, Methods Enzymol. 155: 558-568), PCR-based overlap extension (Ho *et al.*, 1989, Gene 77: 51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques 8: 404-407), *etc.* Modifications can be confirmed by double stranded dideoxynucleotide DNA sequencing.

In certain embodiments, a nucleic acid encoding a secretory form of the hsp of choice is used to practice the methods of the present invention. Such a nucleic acid can be constructed by deleting the coding sequence for the ER retention signal, KDEL. Optionally, the KDEL coding sequence is replaced with a molecular tag to facilitate the recognition and purification of the hsp, such as the Fc portion of murine IgG1. U.S. Application Serial No. 09/253,439 demonstrates that deletion of the ER retention signal of gp96 resulted in the secretion of gp96-Ig peptide-complexes from transfected tumor cells, and the fusion of the KDEL-deleted gp96 with murine IgG1 facilitated its detection by ELISA and FACS analysis and its purification by affinity chromatography with the aid of Protein A.

4.4.2. EXPRESSION SYSTEMS

Nucleotide sequences encoding an hsp can be inserted into the expression vector for propagation and expression in recombinant cells. An expression construct, as

used herein, refers to a nucleotide sequence encoding an hsp operably associated with one or more regulatory regions which allows expression of the hsp in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the hsp polypeptide sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation of the hsp sequence. A variety of expression vectors may be used for the expression of hsps, including, but not limited to, plasmids, cosmids, phage, phagemids, or modified viruses. Examples include bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). Typically, such expression vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the hsp gene sequence, and one or more selection markers.

For expression of hsps in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the Hsp70 gene (Williams *et al.*, 1989, Cancer Res. 49: 2735-42 ; Taylor *et al.*, 1990, Mol. Cell. Biol. 10: 165-75).

The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can also be used for the recombinant expression of hsps in cells of a particular tissue type: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38: 639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50: 399-409; MacDonald, 1987, Hepatology 7: 425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315: 115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38: 647-658; Adames *et al.*, 1985, Nature 318: 533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7: 1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45: 485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes Dev. 1: 268-276), alpha-fetoprotein gene control region which is active in liver

(Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5: 1639-1648; Hammer *et al.*, 1987, Science 235: 53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes Dev. 1: 161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315: 338-340; Kollias *et al.*, 1986, Cell 46: 89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48: 703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314: 283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234: 1372-1378).

The efficiency of expression of the hsp in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153: 516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1: 36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an hsp. For long term, high yield production of hsps, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. U.S.A. 48: 2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22: 817) genes can be employed in *tk*$^-$, *hgprt*$^-$ or *aprt*$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. U.S.A. 77: 3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. U.S.A. 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, 1981, Proc. Natl. Acad.

Sci. U.S.A. 78: 2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150: 1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30: 147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

In order to insert the hsp coding sequence into the cloning site of a vector, DNA sequences with regulatory functions, such as promoters, must be attached to hsp coding sequences. To do this, linkers or adapters providing the appropriate compatible restriction sites may be ligated to the ends of cDNA or synthetic DNA encoding an hsp, by techniques well known in the art (Wu *et al.*, 1987, Methods Enzymol. 152: 343-349). Cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. Alternatively, a desired restriction enzyme site can be introduced into a fragment of DNA by amplification of the DNA by use of PCR with primers containing the desired restriction enzyme site.

The expression construct comprising an hsp coding sequence operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of the hsp-peptide complexes of the invention without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the hsp coding sequence into the genome of the host cell, *e.g.*, via homologous recombination. In this instance, it is not necessary to employ an expression vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the hsp in the host cells.

Expression constructs containing cloned hsp coding sequences can be introduced into the mammalian host cell by a variety of techniques known in the art, including but not limited to calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11: 223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215: 166-168), electroporation (Wolff *et al.*, 1987, Proc. Natl. Acad. Sci. 84: 3344), and microinjection (Cappechi, 1980, Cell 22: 479-488).

For long term, high yield production of properly processed hsp-peptide complexes, stable expression in mammalian cells is preferred. Cell lines that stably express hsps to produce the hsp-peptide complexes of the present invention may be engineered by using a vector that contains a selectable marker. By way of example but not limitation,

following the introduction of the expression constructs, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the expression construct confers resistance to the selection and optimally allows cells to stably integrate the expression construct into their chromosomes and to grow in culture and to be expanded into cell lines. Such cells can be cultured for a long period of time while the hsp is expressed continuously.

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by techniques well known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

Alternatively, number of viral-based expression systems may also be utilized with mammalian cells for recombinant expression of hsps. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17: 725), adenovirus (Van Doren *et al.*, 1984, Mol. Cell Biol. 4: 1653), adeno-associated virus (McLaughlin *et al.*, 1988, J. Virol. 62: 1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc. Natl. Acad. Sci. 79: 4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control region, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts (see, *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. U.S.A. 81: 3655-3659).

Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been developed for recombinant gene expression which exist as stable, multicopy (20-300 copies/cell) extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a

promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene construct is transfected into cultured mammalian cells, for example, by the techniques of calcium phosphate coprecipitation or electroporation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant selectable marker, such as histidinol and G418 resistance. For example, BPV vectors such as pBCMGSNeo and pBCMGHis may be used to express hsps (Karasuyama *et al.*, Eur. J. Immunol. 18: 97-104; Ohe *et al.*, Human Gene Therapy 6: 325-33) which may then be transfected into a diverse range of cell types for hsp expression.

Alternatively, the vaccinia 7.5K promoter may be used (see, e.g., Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 7415-7419; Mackett *et al.*, 1984, J. Virol. 49: 857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A. 79: 4927-4931) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) may be used. Such vectors can be used with a broad range of human host cells, e.g., EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot. Eng. Tech. 2: 14-18), pDR2 and λDR2 (available from Clontech Laboratories).

Recombinant hsp expression can also be achieved by a retrovirus-based expression system. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. In retroviruses such as Moloney murine leukemia virus, most of the viral gene sequences can be removed and replaced with an hsp coding sequence, while the missing viral functions can be supplied in *trans*. The host range for infection by a retroviral vector can also be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The ND-associated antigenic peptide DNA is inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple

drug selection markers may also be included in the expression vector to facilitate selection of infected cells (see McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38: 91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18: 3587-3596; Choulika *et al.*, 1996, J. Virol 70: 1792-1798; Boesen *et al.*, 1994, Biotherapy 6: 291-302; Salmons and Gunzberg, 1993, Human Gene Therapy 4: 129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3: 110-114).

Any cell type that can endogenously produce an ND-associated antigenic polypeptide can be used for recombinant hsp expression. The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, a cells may be cultured under conditions emulating the nutritional and physiological requirements of a cell in which the ND-associated antigen is endogenously expressed. Modified culture conditions and media may be used to enhance production of hsp–peptide complexes. For example, recombinant cells may be grown under conditions that promote inducible hsp expression. Any technique known in the art may be applied to establish the optimal conditions for producing ND-associated hsp–peptide complexes.

4.5. FORMATION OF COVALENT ND-ASSOCIATED HSP-PEPTIDE COMPLEXES

As an alternative to non-covalent complexes of hsps in association with ND-associated antigenic peptides, ND-associated antigenic peptides covalently attached to hsps may be used as vaccines to elicit an immune response. Hsp-peptide complexes are preferably cross-linked after their purification from cells or tissues as described in Section 4.3, *supra*. In one embodiment, hsps are covalently coupled to ND-associated antigen by chemical crosslinking. Chemical crosslinking methods are well known in the art. For example, in a preferred embodiment, glutaraldehyde crosslinking may be used. Glutaradehyde crosslinking has been used for formation of covalent complexes of peptides and hsps (see Barrios *et al.*, 1992, Eur. J. Immunol. 22: 1365-1372). Preferably, 1-2 mg of ND-associated hsp-peptide complex is crosslinked in the presence of 0.002% glutaraldehyde for 2 hours. Glutaraldehyde is removed by dialysis against phosphate buffered saline (PBS) overnight (Lussow *et al.*, 1991, Eur. J. Immunol. 21: 2297-2302).

In another embodiment, the hsp and ND-associated antigen(s) are crosslinked by ultraviolet (UV) crosslinking.

4.6. DETERMINATION OF IMMUNOGENICITY OF ND-ASSOCIATED HSP- PEPTIDE COMPLEXES

Optionally, the ND-associated heat shock protein-peptide complexes can be assayed for immunogenicity using any method known in the art. By way of example but not limitation, one of the following three procedures can be used.

4.6.1. THE MLTC ASSAY

Briefly, mice are injected with the hsp ND-associated antigen complex, using any convenient route of administration. As a negative control, other mice are injected with heat shock protein peptide complexes not associated with ND-associated antigens, or cells containing heat shock protein peptide complexes not associated with ND-associated antigens. Cells containing ND-associated antigens may act as a positive control for the assay. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be re-stimulated subsequently *in vitro* by the addition of dead cells that expressed the antigen of interest.

For example, $8x10^6$ immune spleen cells may be stimulated with $4x10^4$ mitomycin C treated or γ-irradiated (5-10,000 rads) cells containing the antigen of interest (or cells transfected with an appropriate gene, as the case may be) in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant may be included in the culture medium as a source of T cell growth factors (*See,* Glasebrook, et al., 1980, *J. Exp. Med.* 151:876). To test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice may also be re-stimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay (*See,* Palladino, et al., 1987, *Cancer Res.* 47:5074-5079 and Blachere, at al., 1993, *J. Immunotherapy* 14:352-356). In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The

target cells are prelabelled by incubating $1x10^6$ target cells in culture medium containing 20 mCi ^{51}Cr/ml for one hour at 37°C. The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous ^{51}Cr release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelletted by centrifugation at 200g for 5 minutes. The amount of ^{51}Cr released into the supernatant is measured by a gamma counter. The percent cytotoxicity is measured as cpm in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a final concentration of 12.5%.

4.6.2. CD4+ T CELL PROLIFERATION ASSAY

Primary T cells are obtained from spleen, fresh blood, or CSF and purified by centrifugation using FICOLL-PAQUE PLUS (Pharmacia, Upsalla, Sweden) essentially as described by Kruse and Sebald, 1992, EMBO J. 11: 3237-3244. The peripheral blood mononuclear cells are incubated for 7-10 days with a lysate of cells expressing an ND-associated antigen. Antigen presenting cells may, optionally be added to the culture 24 to 48 hours prior to the assay, in order to process and present the antigen in the lysate. The cells are then harvested by centrifugation, and washed in RPMI 1640 media (GibcoBRL, Gaithersburg, Md.). $5x10^4$ activated T cells/well (PHA-blasts) are in RPMI 1640 media containing 10% fetal bovine serum, 10 mM HEPES, pH 7.5, 2 mM L-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulphate in 96 well plates for 72 hrs at 37°C., pulsed with 1 μCi ^3H-thymidine (DuPont NEN, Boston, Mass.)/well for 6 hrs, harvested, and radioactivity measured in a TOPCOUNT scintillation counter (Packard Instrument Co., Meriden, Conn.).

4.6.3. ANTIBODY RESPONSE ASSAY

In a certain embodiment of the invention, the immunogenicity of a ND-associated hsp-peptide complex is determined by measuring antibodies produced in response to the vaccination with the complex. In one mode of the embodiment, microtitre

plates (96-well Immuno Plate II, Nunc) are coated with 50 µl/well of a 0.75 µg/ml solution of a purified, non-hsp-complexed form of the peptide used in the vaccine (*e.g.* Aβ42) in PBS at 4°C for 16 hours and at 20°C for 1 hour. The wells are emptied and blocked with 200 µl PBS-T-BSA (PBS containing 0.05% (v/v) TWEEN 20 and 1% (w/v) bovine serum albumin) per well at 20°C for 1 hour, then washed 3 times with PBS-T. Fifty µl/well of plasma or CSF from a vaccinated animal (such as a model mouse or a human patient) is applied at 20°C for 1 hour, and the plates are washed 3 times with PBS-T. The anti-peptide antibody activity is then measured calorimetrically after incubating at 20°C for 1 hour with 50µl/well of sheep anti-mouse or anti-human immunoglobulin, as appropriate, conjugated with horseradish peroxidase (Amersham) diluted 1:1,500 in PBS-T-BSA and (after 3 further PBS-T washes as above) with 50 µl of an o-phenylene diamine (OPD)-H_2O_2 substrate solution. The reaction is stopped with 150 µl of 2M H_2SO_4 after 5 minutes and absorbance is determined in a Kontron SLT-210 photometer (SLT Lab-instr., Zurich, Switzerland) at 492 nm (ref. 620 nm).

4.7. METHODS FOR DIAGNOSING AND ASSAYING PROGRESS OF AD

There are presently many molecular markers known in the art for the diagnosis of AD (see, *e.g.*, Bancher et al., 1998, J. Neural Transm., Suppl. 53:185-197; Galasko, 1998, J. Neural Transm., Suppl. 53:209-221; issue no. 2 of Neurobiol. Aging, Vol. 19, 1998, including articles by Arai et al., pp. 125-6; Foster, pp. 127-129; Mayeux, pp.139-143; Klunk, pp. 145-157; Hock, pp. 149-151; Robles, pp. 153-157; Hyman, pp. 159-160; and Lannfelt, pp. 165-167) . These methods can be used to determine whether an asymptomatic human subject displays any of the molecular hallmarks of AD. The methods may also be used to diagnose AD in a human subject who exhibits symptoms of the early stages of AD. These methods are useful for identifying individuals at risk of AD who would benefit from the methods of treatment and prevention of AD of the invention. Finally, the methods can also be used to assay the efficacy of the vaccines of the present invention and monitor the progress of AD in those receiving the vaccines. The diagnostic methods for AD to be utilized according to the present invention include but are not limited to testing for molecular indicators of AD or for alterations in neurophysiological function that would be affected by AD. In a preferred embodiment, more than one of the assays

described below would be carried out to confirm the diagnosis of the present or extent of AD.

4.7.1. MOLECULAR INDICATORS OF AD

Testing for the presence of phosphorylated Tau protein using a monoclonal antibody specific for the phosphorylated form, as disclosed in U.S. Patent No. 5,733,734, serves as an indicator for AD. In a preferred embodiment, the monoclonal antibody is Alz-50 (U.S. Patent No. 5,811,310). The presence of phosphorylated Tau may be tested in either brain tissue or cerebrospinal fluid or cultures of olfactory neurons from the patient.

Tau proteolysis products have been found to be present in the blood or spinal fluids of individuals with AD (U.S. Patent No. 5,492,812). Thus, testing for the presence of tau peptides in blood or spinal fluid samples may provide a diagnostic measure of the presence or progression of AD in patients and other individuals.

The presence or extent of AD can also be determined by measuring the relative abundance of Aβ42 and Aβ40. In normal individuals, the amount of Aβ40 far exceeds the amount of Aβ42. In contrast, Aβ42 predominates in AD patients. Additionally, all mutations implicated in FAD, whether in the *APP*, *PS1*, or *PS2* genes, relate to the processing of APP, and are thought to produce AD through promoting the synthesis of Aβ42. Thus, the relative amount of Aβ40 and Aβ42, for example in a CSF sample or tissue biopsy from the brain or pancreas from an individual, would be an indicator of the presence or progress of AD in the individual.

Calcium activated neutral proteases are enzymes that regulate signal transduction by modulating the activities of signaling molecules (*e.g.* protein kinases and phosphatases) through partial proteolysis. Calcium activated neutral proteases are themselves regulated by partial proteolysis, wherein in the presence of calcium a precursor form of an enzyme undergoes autoproteolytic cleavage to produce a functional enzyme. It has been shown that the ratios of cleaved to uncleaved calcium activated neutral proteases are altered in AD patients. Thus, by measuring the relative amounts of each isoform in a test subject in comparison with a control subject, it is possible to detect AD in an individual (U.S. Patent No. 5,624,807).

It has been demonstrated that AD patients have elevated levels of acetylcholinesterase (AChE) activity in ocular fluids. A colorimetric assay for the

determination of AChE activity described by Ellman et al. (1961, Biochem. Pharmacol. 7:161-177) may be utilized to measure AchE activity levels in ocular fluid samples, *i.e.* aqueous humor or vitreous humor samples, the result of which would indicate the presence or absence and possibly the extent of AD (U.S. Patent No. 5,595,883).

One of the characteristics of AD is an impairment in cytokine secretion, for example IL-1, IL-3 and IL-6. It has been postulated that the impairment is a downstream effect of impaired neural function. Thus it would be possible to assay for blood cytokine levels as indicators of AD (U.S. Patent No. 5,874,312)

Unless indicated otherwise, the proteins or peptides described *supra* may be assayed for by a radioimmunoassay, an enzyme-linked immunosorbant assay, a sandwich assay, a gel immunodiffusion assay, an agglutination assay, a fluorescent immunoassay, a protein A immunoassay or an immunoelectrophoresis assay, or any other method known in the art. These methods are well known to those skilled in the art.

4.7.2. NEUROPHYSIOLOGICAL TESTS

U.S. Patent No.5,778,893 discloses methods of diagnosing AD, including the extent of AD, in an individual by applying agents that agonize or antagonize neuromuscular signaling and determining the response of the individual to said agents in comparison to control individuals. For example, a cholinergic antagonist is administered to the eye of an individual, the pupil allowed to dilate in response to the cholinergic antagonist, and the rate of which the pupil returns to its normal diameter measured and compared to the corresponding rates in control individuals.

In an alternative method of measuring neurophysiological output, a light source illuminates the eye of an individual suspected to have AD. The response of the individual's pupils to the light is measured by a computer system connected to a video camera that records the response of the pupils (U.S. Patent 5,883,691).

4.8. METHODS OF USE OF COMPOSITIONS COMPRISING ND-ASSOCIATED HSP-PEPTIDE COMPLEXES

In accordance with the invention, the hsp-based compositions and formulations described in Section 4.3 above and Section 4.8 below, are administered to human patients with NDs. In another embodiment, the compositions and formulations are

administered to human subjects that do not have a ND as a preventative measure from developing the disease. The methods of the present invention can be applied to the preparation of hsp-peptide complexes associated with any neurodegenerative disorder, and compositions comprising the same. Such disorders relate to the central nervous system and/or peripheral nervous system and include, but are not limited to, cognitive and neurodegenerative disorders such as senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, such as spongiform encephalopathies, *e.g.*, Creutzfeldt-Jakob disease, polyglutamine diseases, such as Huntington's disease, myotonic dystrophy, Freidrich's ataxia and other ataxias, well as Gilles de la Tourette's syndrome, autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders that include, but are not limited to schizophrenia, schizoaffective disorder, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, panic disorder, as well as unipolar and bipolar affective disorders. Additional neuropsychiatric and neurodegenerative disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version of which is incorporated herein by reference in its entirety.

In a preferred aspect of the invention, the purified ND associated hsp-peptide complex vaccines may have particular utility in the treatment of human neurodegenerative disorders. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that have similar pathologies.

4.8.1. PREVENTION OF NEURODEGENERATIVE DISORDERS

In certain embodiments, the compositions and formulations of the present invention are administered to a human subject to prevent ND, including inhibiting the progression of a disease in an asymptomatic patient, for example a patient having the molecular landmarks of AD (*e.g.*, above normal levels of phosphorylated Tau and/or Aβ42). In a preferred embodiment, the human subject to which the vaccines of the invention are

administered is one having a genetic background that increases the likelihood of a given ND (*e.g.* having the ϵ4 allele of Apolipoprotein E or having a mutation in APP, PS1 or PS2 which gives rises to FAD). In another embodiment, the human subject to which the preventative vaccines of the invention are administered is a non-senile adult above the age of 60.

4.8.2. TREATMENT OF NEURODEGENERATIVE DISORDERS

In other embodiments, the compositions and formulations of the present invention are administered to a human subject that has been diagnosed with a ND or suspected of having a ND. According to the present invention, treatment of a ND encompasses the treatment of patients already diagnosed as having ND at any clinical stage; the prevention of the disease in the patients with early symptoms and signs; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of a ND; and/or promoting regression of a ND in symptomatic patients.

4.8.3. COMBINATION WITH ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy refers to a therapeutic approach for treating neurodegenerative diseases in which immune cells are administered to a host with the aim that the cells mediate specific immunity, either directly or indirectly, to cells that express ND-associated antigens and/or antigenic components, and result in treatment of the neurodegenerative disorder, or prevention of the neurodegenerative disorder, as the case may be (see U.S. Patent Application Serial No. 08/527,546, filed September 13, 1995, which is incorporated by reference herein in its entirety). The use of hsp-peptide complexes for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997, which is incorporated by reference herein in its entirety. Methods for sensitizing antigen presenting cells (APC) using hsps in complexes with antigenic (or immunogenic) molecules, for adoptive immunotherapy are described in detail herein.

According to the invention, therapy by administration of hsp–peptide complexes, using any desired route of administration, is combined with adoptive immunotherapy using APC sensitized with hsp–antigenic molecule complexes. The

hsp–peptide complex-sensitized APC can be administered concurrently with hsp–peptide complexes, or before or after administration of hsp–peptide complexes. Furthermore, the mode of administration can be varied, including but not limited to, *e.g.*, subcutaneously, intravenously, intraperitoneally, intramuscularly, intradermally or mucosally.

The antigen-presenting cells, including but not limited to macrophages, dendritic cells and B-cells, are preferably obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba *et al.*, 1992, J. Exp. Med. 176:1693-1702. APC can be obtained by any of various methods known in the art. In a preferred aspect human macrophages are used, obtained from human blood cells.

By way of example, but not limitation, macrophages can be obtained as follows: Mononuclear cells are isolated from peripheral blood of a patient (preferably the patient to be treated), by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The cells are incubated at 37°C for 1 hr, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1 mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF); increased numbers of dendritic cells may be obtained by incubating with granulocyte-macrophage-colony stimulating factor (GM-CSF) as described in detail by Inaba, K., *et al.*, 1992, J. Exp. Med. 176:1693-1702.

APC are sensitized with hsps bound to ND-associated antigenic molecules by incubating the cells *in vitro* with the complexes. The APC are sensitized with complexes of hsps and antigenic molecules preferably by incubating *in vitro* with the hsp–complex at 37°C for 15 minutes to 24 hours. By way of example but not limitation, $4x10^7$ macrophages can be incubated with 10 microgram gp96-peptide complexes per ml or 100 microgram hsp90-peptide complexes per ml at 37°C for 15 minutes-24 hours in 1 ml plain RPMI medium. The cells are washed three times and resuspended in a physiological medium preferably sterile, at a convenient concentration (*e.g.*, $1x10^7$/ml) for injection in a patient. In a preferred embodiment, the antigen presenting cells are autologous to the

patient, that is, the patient into which the sensitized APCs are injected is the patient from which the APC were originally isolated,.

Optionally, the ability of sensitized APC to stimulate, for example, the antigen-specific, class I-restricted cytotoxic T-lymphocytes (CTL) can be monitored by their ability to stimulate CTLs to release tumor necrosis factor, and by their ability to act as targets of such CTLs.

The hsp–antigenic molecule-sensitized APC are reinfused into the patient systemically, preferably intravenously, by conventional clinical procedures. These activated cells are reinfused, preferentially by systemic administration into the autologous patient. Patients generally receive from about 10^6 to about 10^{12} sensitized macrophages, depending on the condition of the patient. In some regimens, patients may optionally receive in addition a suitable dosage of a biological response modifier including but not limited to the cytokines IFN-α , IFN-γ, IL-2, IL-4, IL-6, TNF or other cytokine growth factor.

4.8.4. PASSIVE IMMUNOTHERAPY

ND associated hsp-peptide complexes can also be used for passive immunotherapy against neurodegenerative disorders. Passive immunity is the short-term protection of a host, achieved by the administration of pre-formed antibody directed against a heterologous organism. For example, ND associated hsp-peptide complexes may used to elicit an immune response in a subject, the sera removed from the subject and used for treatment or prevention of a neurodegerative disorder in a subject having a disorder caused by the presence of a common antigen.

4.9. DOSAGE REGIMENS

Hsp-antigenic molecule complexes are administered to human subjects in doses in a range of about 1 μg to about 5000 μg, preferably in a range of about 1 μg to about 1500 μg.

Methods of introduction include but are not limited to intrathecal, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The hsps-peptide complexes may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or

mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local; this may be achieved, for example and not by way of limitation, by topical application, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In a specific embodiment, the hsp compositions of the present invention are administered intrathecally by an implant be placed in or near the lesioned area of the nervous system. Suitable implants include, but are not limited to, gelfoam, wax, liposome or microparticle-based implants. Such compositions are preferably used when it is desired to achieve sustained release of the hsp-peptide complexes.

In a specific embodiment, the hsp compositions of the present invention are administered into the cerebrospinal fluid (CSF) of a patient by means of injection. In certain modes of the embodiment, administration is in or near the lesioned area of the nervous system.

In another specific embodiment, the hsp compositions are administered, either intradermally or subcutaneously, with sites of administration varied sequentially. For example, and not by way of limitation, the doses recited above are given once weekly for a period of about 4 to 6 weeks, and the mode of administration is varied with each administration. Each site of administration may be varied sequentially. Thus, by way of example and not limitation, the injections can be given, either intradermally or subcutaneously, locally or at a site distant from the brain or central nervous system. The same site can be repeated after a gap of one or more injections. Also, split injections can be given. Thus, for example, half the dose can be given in one site and the other half in another site on the same day.

After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one month. Later injections can be given monthly. The pace of later injections can be modified, depending upon the patient's clinical progress and responsiveness to the therapy. Alternatively, the mode of administration is sequentially varied, *e.g.*, weekly administrations are given in sequence intradermally or subcutaneously.

4.10. VACCINE FORMULATIONS

The ND-associated hsp-peptide complexes, in accordance with the invention, can be formulated into pharmaceutical preparations for administration to mammals, preferably humans, as preventative or therapeutic vaccines for a ND. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier can be prepared, packaged, and labeled for prevention and/or therapy of the ND for which the ND-associated hsp-peptide complexes are prepared.

If the complex is water-soluble, then it can be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it can be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the compounds and their physiologically acceptable solvates can be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral, rectal administration.

For oral administration, the pharmaceutical preparation can be in liquid form, for example, solutions, syrups or suspensions, or can be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets can be coated by methods well-known in the art.

Preparations for oral administration can be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions can take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds can be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds can be formulated into creams, lotions, ointments or tinctures, *e.g.*, containing conventional bases, such as hydrocarbons, petrolatum, lanolin, waxes, glycerin, or alcohol. The compounds can also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (*e.g.*, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The compositions can, if desired, be presented in a pack or dispenser device which can contain one or more unit dosage forms containing the active ingredient. The pack can for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration.

4.11. KITS

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically effective amounts of the ND-associated hsp-peptide complexes in pharmaceutically acceptable form. The ND-derived hsp-peptide complex in a vial of a kit of the invention can be in the form of a pharmaceutically acceptable solution, *e.g.*, in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex can be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (*e.g.*, saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of ND-derived hsp-antigenic molecule complexes by a clinician or by the patient.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

2. The composition of claim 1, wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

3. A composition of claim 2, wherein the antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder displays the antigenicity of an antigen associated with Alzheimer's Disease.

4. The composition of claim 3, wherein the population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from an individual with Alzheimer's Disease.

5. The composition of claim 4, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from brain tissue.

6. The composition of claim 4, wherein the purified population of complexes of

heat shock proteins bound to endogenous antigenic molecules is isolated from pancreatic tissue.

7. The composition of claim 3, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from brain or pancreatic tissue of an individual with trisomy 21.

8. The composition of claim 4 or 7, wherein the individual is deceased.

9. The composition of claim 4,wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from a culture of olfactory neurons isolated from said individual.

10. The composition of claim 3, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from an Alzheimer's Disease model organism.

11. The composition of claim 10, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from brain or pancreatic tissue.

12. The composition of claim 10, wherein the organism is a rat, said rat having received infusions of Aβ and TGF-β.

13. The composition of claim 10, wherein the organism is a transgenic mouse.

14. The composition of claim 13, wherein the mouse expresses human APP V717F.

15. The composition of claim 14, wherein the mouse expresses a second antigenic molecule, which second antigenic molecule displays the antigenicity of an antigen associated with Alzheimer's Disease.

16. The composition of claim 10, wherein the organism is a non-human primate.

17. The composition of claim 3, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from a tissue or cell culture model system for Alzheimer's disease.

18. The composition of claim 17, wherein the cell culture cells are selected from the group consisting of immortalized murine trisomy 16 neuronal cells, mouse neuroblastoma cell line S20Y, and mouse embryonal carcinoma cell line P19.

19. The composition of claim 3, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from amyloid plaques or cells containing neurofibrillary tangles.

20. The composition of claim 1, wherein the heat shock protein is an hsp70.

21. The composition of claim 1, wherein the heat shock protein is an hsp90.

22. The composition of claim 1, wherein the heat shock protein is a gp96.

23. The composition of claim 1, wherein the heat shock protein is calreticulin.

24. The composition of claim 1, further comprising a pharmaceutically acceptable carrier.

25. The composition of claim 1, wherein the complexes are present in an amount effective to treat or prevent said neurological disorder.

26. The composition of claim 3, wherein the complexes are present in an amount effective to treat or prevent Alzheimer's Disease.

27. The composition of claim 1, wherein said heat shock proteins are covalently linked to said antigenic molecules.

28. The composition of claim 1, wherein said heat shock proteins are non-covalently linked to said antigenic molecules.

29. The composition of claim 1, wherein the population of complexes of heat shock proteins bound to endogenous antigenic molecules is purified to apparent homogeneity, as viewed on an SDS-PAGE gel.

30. A pharmaceutical composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

31. A method of treating or preventing a neurodegenerative disorder in a mammal comprising administering to the mammal a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

32. The method of claim 31, wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS

dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

33. The method of claim 32, wherein the antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder displays the antigenicity of an antigen associated with Alzheimer's Disease.

34. The method of claim 33, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from an individual with Alzheimer's Disease.

35. The method of claim 34, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from brain tissue.

36. The method of claim 34, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from pancreatic tissue.

37. The method of claim 33, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from an individual with trisomy 21.

38. The method of claim 34 or 37, wherein the individual is deceased.

39. The method of claim 34, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from a culture of olfactory neurons isolated from said individual.

40. The method of claim 39, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is autologous to the mammal.

41. The method of claim 39, wherein the olfactory neurons originate from a

cadaver of an individual having Alzheimer's Disease or trisomy 21.

42. The method of claim 33, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from an Alzheimer's Disease model organism.

43. The method of claim 42, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from brain or pancreatic tissue.

44. The method of claim 42, wherein the organism is a rat, said rat having received infusions of Aβ and TGF-β.

45. The method of claim 42, wherein the organism is a transgenic mouse.

46. The method of claim 45, wherein the mouse expresses human APP V717F.

47. The method of claim 46, wherein the mouse expresses a second antigenic molecule, which second antigenic molecule displays the antigenicity of an antigen associated with Alzheimer's Disease.

48. The method of claim 42, wherein the organism is a non-human primate.

49. The method of claim 33, wherein the purified population of complexes of heat shock proteins bound to endogenous antigenic molecules is isolated from a tissue or cell culture model system for Alzheimer's Disease.

50. The method of claim 49, wherein the cell culture cells are selected from the group consisting of immortalized murine trisomy 16 neuronal cells, mouse neuroblastoma cell line S20Y, and mouse embryonal carcinoma cell line P19.

51. The method of claim 33, wherein the purified population of complexes of

heat shock proteins bound to endogenous antigenic molecules is isolated from amyloid plaques or cells containing neurofibrillary tangles.

52. The method of claim 31, wherein the heat shock protein is an hsp70.

53. The method of claim 31, wherein the heat shock protein is an hsp90.

54. The method of claim 31, wherein the heat shock protein is a gp96.

55. The method of claim 31, wherein the heat shock protein is calreticulin.

56. The method of claim 31, wherein the composition comprising a heat shock protein-peptide complex further comprises a pharmaceutically acceptable carrier.

57. The method of claim 31, wherein the complexes are administered in an amount effective to treat or prevent said neurological disorder.

58. The method of claim 32, wherein the complexes are administered in an amount effective to treat or prevent Alzheimer's Disease.

59. The method of claim 31, wherein said heat shock proteins are covalently linked to said antigenic molecules.

60. The method of claim 31, wherein said heat shock proteins are non-covalently linked to said antigenic molecules.

61. The method of claim 31, wherein the population of complexes of heat shock proteins bound to endogenous antigenic molecules is purified to apparent homogeneity, as viewed on an SDS-PAGE gel.

62. The method of claim 31, wherein the composition is administered intradermally.

63. The method of claim 31, wherein the composition is administered intrathecally.

64. The method of claim 31, further comprising, before, concurrently, or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

65. A kit comprising in one or more separate containers a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder or which population comprises a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

66. The kit of claim 65, wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

67. The kit of claim 66, wherein the neurodegenerative disorder is Alzheimer's Disease.

68. The kit of claim 65, further comprising instructions for administration to humans.

69. A method of treating or preventing a neurodegenerative disorder in a subject having a neurodegenerative disorder or in whom treatment or prevention of a neurodegenerative disorder is desired comprising:

a) isolating or culturing a mammalian tissue or cell that displays a pathology associated with a neurodegenerative disorder or expresses an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder;

b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules; and

c) administering to the subject an amount of the recovered complexes effective to treat or protect against the neurodegenerative disorder.

70. The method of claim 69, further comprising, after step (b) and before step (c), the step of treating the complexes with a crosslinking agent such that the heat shock proteins become covalently associated with the antigenic molecules.

71. A method for eliciting in an individual an immune response against a tissue or cell which displays a pathology associated with a neurodegenerative disorder comprising administering to the individual a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said complexes being purified from mammalian tissues or cells, which tissues or cells display a pathology associated with a neurodegenerative disorder.

72. A method for eliciting in an individual an immune response against an antigen associated with a neurodegenerative disorder comprising administering to the individual a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules, said population being purified from

mammalian tissues or cells and comprising a complex in which the antigenic molecule displays the antigenicity of an antigen associated with a neurodegenerative disorder.

73. The method of claim 71 or 72 wherein the neurodegenerative disorder is Alzheimer's Disease, age-related loss of cognitive function, senile dementia, Parkinson's disease, amyotrophic lateral sclerosis, Wilson's Disease, cerebral palsy, progressive supranuclear palsy, Guam disease, Lewy body dementia, prion diseases, spongiform encephalopathies, Creutzfeldt-Jakob disease, polyglutamine diseases, Huntington's disease, myotonic dystrophy, Freidrich's ataxia, ataxia, Gilles de la Tourette's syndrome, seizure disorders, epilepsy, chronic seizure disorder, stroke, brain trauma, spinal cord trauma, AIDS dementia, alcoholism, autism, retinal ischemia, glaucoma, autonomic function disorder, hypertension, neuropsychiatric disorder, schizophrenia, or schizoaffective disorder.

74. A method for preparing a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules associated with a neurodegenerative disorder, comprising:

a) isolating or culturing a mammalian tissue or cell that expresses an antigenic molecule that displays the antigenicity of an antigen associated with a neurodegenerative disorder; and

b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules.

75. A method for preparing a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules associated with a neurodegenerative disorder, comprising:

a) isolating or culturing a mammalian tissue or cell that displays a pathology associated with a neurodegenerative disorder; and

b) recovering a population of complexes of the heat shock proteins noncovalently associated with the antigenic molecules.

76. The method of claim 74 or 75, wherein the mammalian tissue or cell is cultured.

77. The method of claim 76, wherein the mammalian tissue or cell is transformed with a nucleic acid encoding a heat shock protein operatively linked to a promoter and growing the tissue or cell under conditions such that the heat shock protein is expressed by said tissue or cell.

INTERNATIONAL SEARCH REPORT

Inten ication No.
PCT/US01/01671

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00, 39/00; C07K 2/00, 4/00, 14/00, 17/00
US CL : 424/184.1, 193.1, 194.1; 435/325; 514/2, 21; 530/300, 350, 403
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/184.1, 193.1, 194.1; 435/325; 514/2, 21; 530/300, 350, 403

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST, DIALOG, MEDLINE (search terms: author name, heat shock/stress protein, gp96, Alzheimer's disease, antigen)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BARRIOS et al. Heat shock proteins as carrier molecules: in vivo help effect mediated by Escherichia coli GroL and DnaL proteins requires cross-linking with antigen. Clin. Exp. Immunol. 1994, Vol 98, pages 229-233, see entire document.	1-68, 74, 76-77
A	BRELOER et al. Isolation of processed, H-2K-binding ovalbumin-derived peptides associated with the stress proteins HSP70 and GP96. Eur. J. Immunol. 1998, Vol 28, pages 1016-1021, see entire document.	1-68, 74, 76-77
A	MENORET et al. Association of peptides with heat shock protein gp96 occurs in vivo and not after cell lysis. Biochem. Biophys. Res. Comm., 1999, Vol 262, pages 813-818, see entire document.	1-68, 74, 76-77
A	MORIMOTO et al. Stress-inducible responses and heat shock proteins: new phamacological targets for cytoprotection. Nature Biotech. 1998, Vol 16, pages 833-838, see entire document.	1-68, 74, 76-77
A	NIELAND et al. Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein gp96/GRP94. Proc. Natl. Acad. Sci. USA 1996, Vol 93, pages 6135-6139, see entire document.	1-68, 74, 76-77
A	PAPPOLLA et al. The heat shock/oxidative stress connection. Mol. Chem. Neuropath. 1996, Vol 28, pages 21-34, especially pages 24-30.	1-68, 74, 76-77

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 2001 (16.04.2001)	13 JUN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Bridget E. Bunner Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

Inter application No.
PCT/US01/01671

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUTO et al. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science 1995, Vol 269. No. 5230, pages 1585-1588, see entire document.	1-68, 74, 76-77
A, P	WYTTENBACH et al. Effects of heat shock protein 40 (HDJ-2) and proteosome inhibition on protein aggregation in cellular models of Huntington's disease. Proc. Natl. Acad. Sci. USA 2000, Vol 97. No. 6, pages 2898-2903, see entire document.	1-68, 74, 76-77

INTERNATIONAL SEARCH REPORT

Inter al application No.
PCT/US01/01671

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: Please See Continuation Sheet

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Int ial application No.
PCT/US01/01671

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. Claims 1-68, 74, and 76-77, drawn to a composition comprising a population of complexes of heat shock proteins bound to antigenic molecules associated with a neurodegenerative disorder, a method of treating or preventing a neurodegenerative disorder comprising administering the composition, and a method of preparing the composition.

II. Claims 69-70, drawn to a method of treating or preventing a neurodegenerative disorder comprising isolating or culturing a tissue or cell that displays a pathology associated with a neurodegenerative disorder and administering a complex isolated therefrom.

III. Claims 71-73, drawn to a method of eliciting an immune response against a cell or tissue by administering a composition of heat shock proteins bound to antigenic molecules, wherein the complexes are purified from mammalian tissues or cells.

IV. Claims 75-77, in part, drawn to a method for preparing a composition comprising a purified population of complexes of heat shock proteins bound to endogenous antigenic molecules associated with a neurodegenerative disorder.

The inventions listed as Groups I, II, III, and IV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Unity of Invention is lacking for Groups I-IV because the PCT Rules do not provide for the search and examination of more than one method of use and one method of making for the first claimed product.
This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.
In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

A. an individual with Alzheimer's disease
B. an individual with trisomy 21
C. a rat with amyloid beta peptide and TGF-beta infusions
D. a transgenic mouse expressing human APP V717F
E. a non-human primate
F. a cell/tissue culture model system for Alzheimer's disease
G. amyloid plaques or cells containing neurofibrillary tangles

The following claim(s) are generic: 1-3, 10-11, 20-33, 42-43, and 52-77.
The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:
The special technical feature of (A) is a patient population suffering from Alzheimer's disease. This special technical feature is not shared by any of the other species.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.
In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

H. hsp70
I. hsp90
J. gp96
K. calreticulin

The following claim(s) are generic: 1-19, 24-51, and 56-77.
The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:
The special technical feature of (H) is hsp70 in the recited complex. None of the other species recite this special technical feature.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.
In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

L. brain tissue
M. pancreatic tissue
N. olfactory neurons
O. amyloid plaques
P. cells containing neurofibrillary tangles
Q. immortalized murine trisomy neuronal cells
R. mouse neuroblastoma cell line S20Y
S. mouse embryonal carcinoma cell line P19

The following claim(s) are generic: 7-8, 10, 12-16, 20-33, 37-38, 40-42, 44-49, and 52-77.
The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: